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Heterodimer formation is essential for heparanase enzymatic activity☆

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Abstract

Heparanase is an endo-β-D-glucuronidase involved in cleavage of heparan sulfate residues and hence participates in extracellular matrix degradation and remodeling. The heparanase cDNA encodes for a polypeptide of 543 amino acids that appears as a ~65 kDa band in SDS–PAGE analysis. The protein undergoes a proteolytic cleavage that is likely to occur at two potential cleavage sites, Glu^{109}–Ser^{110} and Gln^{157}–Lys^{158}, yielding an 8 kDa polypeptide at the N-terminus, a 50 kDa polypeptide at the C-terminus, and a 6 kDa linker polypeptide that resides in-between. The active form of heparanase has long been thought to be a 50 kDa polypeptide isolated from cells and tissues. However, attempts to obtain heparanase activity after expression of the 50 kDa polypeptide failed, suggesting that the N-terminal region is important for heparanase enzymatic activity. It has been hypothesized that heterodimer formation between the 8 and 50 kDa heparanase subunits is important for heparanase enzymatic activity. By individually or co-expressing the 8 and 50 kDa heparanase subunits in mammalian cells, we demonstrate specific association between the heparanase subunits by means of co-immunoprecipitation and pull-down experiments. Moreover, a region in the 50 kDa heparanase subunit that mediates interaction with the 8 kDa subunit was identified. Altogether, our results clearly indicate that heterodimer formation is necessary and sufficient for heparanase enzymatic activity in mammalian cells.
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Heparan sulfate (HS) side chains of heparan sulfate proteoglycans (HSPGs) bind a wide variety of proteins including major components of the extracellular matrix (ECM) such as collagen IV, laminin, and fibronectin, and thereby play an important role in ECM organization, self-assembly, and insolubility [1–3]. Moreover, by binding a multitude of proteins, HSPGs ensure that bioactive molecules such as growth factors, chemokines, lipoproteins, and enzymes are localized to the cell surface and ECM and function in the control of normal and pathological processes, among which are morphogenesis, tissue repair, inflammation, autoimmunity, tumor metastasis, and angiogenesis [1–3]. Therefore, cleavage of HSPGs is likely to release potent regulators that can alter the functional state of tissues and provide a mechanism by which cells can respond rapidly to changes in the extracellular environment.

Heparanase is an endo-β-D-glucuronidase involved in cleavage of HS residues and hence participates in ECM degradation and remodeling. Heparanase activity has been traditionally correlated with the metastatic potential of tumor-derived cell types [4–7]. Similarly, heparanase has been shown to facilitate cell invasion associated with autoimmunity, inflammation, and angiogenesis [5–8]. More recently, heparanase upregulation was detected in a variety of human primary tumors correlating, in some cases, with increased tumor vascularity and poor postoperative survival [9,10].

The cloning of a single human heparanase cDNA sequence was independently reported by several groups [11–14], suggesting that one dominant HS-degrading

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endoglycosidase is expressed by mammalian cells. This stands in contrast with the large number of proteases that can degrade and remodel polypeptides in the ECM [15], positioning heparanase as a potentially new and promising drug target. The heparanase cDNA encodes for a polypeptide of 543 amino acids that appears as a ~65 kDa band in SDS–PAGE analysis. The protein undergoes proteolytic processing which is likely to occur at two potential cleavage sites, Glu^{109}–Ser^{110} and Gln^{157}–Lys^{158}, yielding an 8 kDa polypeptide at the N-terminus, a 50 kDa polypeptide at the C-terminus, and a 6 kDa linker polypeptide that resides in-between [5,6,16]. Heparanase activity is readily obtained after transfection of mammalian cells with plasmid vectors encoding the full-length heparanase cDNA [11–14]. The active form of heparanase has long been thought to be a 50 kDa polypeptide, isolated from cells and tissues. However, attempts to obtain heparanase activity after expression of the 50 kDa polypeptide failed [5–7], suggesting that the N-terminal region is important for heparanase enzymatic activity. It was noted that the purified active heparanase enzyme appears in SDS–PAGE analysis as a 50 kDa protein that was accompanied by an 8 kDa protein, an observation that raised the hypothesis that the enzyme exists as a heterodimer composed of the 50 kDa subunit (Lys^{158}–Ile^{543}) non-covalently associated with the 8 kDa peptide (Gln^{36} to Glu^{109}) [16,17]. The present study was performed to elucidate whether heterodimer formation between the 8 and 50 kDa heparanase subunits is important for heparanase enzymatic activity in mammalian cells. By individually, or co-expressing the 8 and 50 kDa heparanase subunits in mammalian cells, we demonstrate the association between the heparanase subunits by means of co-immunoprecipitation (IP) and pull-down experiments and identified a region in the 50 kDa protein that interacts with the 8 kDa subunit. Moreover, we show that heterodimer formation is necessary and sufficient for heparanase enzymatic activity.

Materials and methods

Antibodies and reagents. Anti-8 kDa antibodies were raised against a peptide (G^{96}TKTDFLIFDPKK^{108}) located at the C-terminus of the 8 kDa subunit (anti-8). Anti-50 kDa antibodies were raised against a peptide (R^{273}KTAKMLKSFLKAGGEVI^{290}) located in the 50 kDa subunit (anti-50) [16]. These peptides were conjugated to keyhole limpet hemocyanin (KLH) and KLH-conjugated peptides were injected into rabbits [16]. Antibody specificity was verified by immunoblot analysis. Anti-c-Myc epitope (sc-40) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Complete protease inhibitor tablets were purchased from Roche (Mannheim, Germany). Glutathione–Sepharose beads and B-PER reagent are products of Pierce (Rockford, IL). pSecTag2 vector was purchased from Invitrogen (Carlsbad, CA) and pGEX-4 T-3 was purchased from Pharmacia–Amersham. Pfu DNA polymerase and T4 Liga Fast are products of Promega (Madison, WI).

Cloning and expression of heparanase subunits in mammalian cells. The pcDNA3 plasmid containing the full-length human heparanase cDNA construct was used as the PCR template for generation of the 8 and 50 kDa constructs. Primers used are as follows- 8F: 5′-GGA-ATT-CAG-GAC-GTC-GTG-GAC-CTG-3′and 8R: 5′-GC-CGC-TCG-AGA-TTC-CTT-CTT-GGG-ATC-GAA-AAT-3′ for amplification of the 8 kDa (Gln^{36}–Glu^{109}) subunit, and primers 50F: 5′-GGA-ATT-CA G-AAA-AAG-TTC-AAG-AAC-AGC-A-3′ and 50R: 5′-GC-CGC-TC G-AGA-GAT-GCA-AGC-AGC-AAC-TTT-GG-3′ for amplification of the 50 kDa subunit (Lys^{158}–Ile^{543}). Primers 50F: 5′-GGA-ATT-CAG-AAA-AAG-TTC-AAG-AAC-AGC-A-3′ and NTR: 5′-GC-CG C-TCG-AGC-TCC-ACC-AGC-CTT-CTG-GAA-3′ were used for amplification of the N-terminus (NT, Lys^{158}–Gly^{287}) fragment. Primers MDF: 5′-GGA-ATT-CGA-GAA-GTG-ATT-GAT-TCA-GTT-AC-3′ and MDR: 5′-GC-CGC-TCG-AGC-CTT-GGT-GCC-CAC-CAA-TT T-C-3′ were used for amplification of the middle (MD, Glu^{288}–Lys^{417}) fragment. Primers CTF: 5′-GGA-ATT-CAG-GTG-TTA-ATG-GCA-AGC-GTG-C-3′ and 50R: 5′-GC-CGC-TCG-AGA-GAT-GCA-AG C-AGC-AAC-TTT-GG-3′ were used for amplification of the C-terminus (CT, Val^{418}–Ile^{543}) fragment. The forward primers contain an inserted *Eco*RI restriction site and the reverse primers an *Xho* restriction site, enabling cloning in-frame into the pSecTag2 vector cloning site. Following PCR with a proofreading enzyme (Pfu-Promega), the vector and constructs were digested with *Eco*RI and *Xho*I, and ligated with T4 ligase. DH5α *Escherichia coli* strain was used for transformation. Clones resistant to ampicillin were propagated in bacteria and evaluated for the presence of the insert by digestion with *Eco*RI/*Xho*I. All PCR amplification products were sequenced to assure correct DNA sequences.

Expression of the 8 kDa heparanase subunit in bacteria. Primer pG.8F (5′-GG-AAT-TCA-CAG-GAC-GTC-GTG-GAC-CTG-3′) and primer pG.8R (5′-GCC-GCT-CGA-GCT-TTC-CTT-CTT-GGG-ATC-GAA-A-3′) were used to amplify the 8 kDa (Gln^{36}–Glu^{109}) fragment. These primers are designed for cloning in-frame at the *Eco*RI/*Xho*I cloning site of pGEX-4T-3 vector that directs expression of GST fused proteins in bacteria. BL21 *E. coli* strain (Pharmacia) was used for protein expression as follows: the bacteria were grown to OD = 0.8, at 26–28 °C, then induced with 0.5 mM IPTG for 3 h, harvested by centrifugation, and subjected to protein purification, as described below.

Heparanase-GST fusion protein purification. Lysis of the bacteria was performed with the B-PER (Pierce) reagent, according to the manufacturer's instructions. The GST-8 kDa fusion protein was found to be mainly concentrated in the pellet of inclusion bodies and could be dissolved by pre-treating the pellet with lysozyme, followed by incubation with 1% Triton X-100 and 0.05% Tween 20 in Tris buffer (50 mM Tris, pH 8, and 150 mM NaCl). The GST-8 kDa fusion protein was purified from the lysates by incubation (18 h, 4 °C) with glutathione immobilized to agarose beads (Pierce), followed by extensive washing with the same buffer. Protein expression and purity were evaluated by SDS–PAGE and Coomassie blue staining.

Cells and transfection. HEK 293 and JAR (human choriocarcinoma) cells were grown in DMEM supplemented with 10% FCS, glutamine, pyruvate, and antibiotics. pSecTag2B vector containing the full-length human heparanase cDNA construct was kindly provided by ImClone Systems (New York, NY). Plasmids were transfected into HEK 293 and JAR cells using Fugene reagent, according to the manufacturer's (Roche) instructions. Transfection proceeded for 48 h followed by selection with 500 μg/ml Zeocin (Invitrogen) for two weeks and stable transfectant pools were further expanded and analyzed. For transient transfection, 293 cells were similarly transfected and harvested after 48 h.

SDS–PAGE and Western blot analysis. Cell extracts were prepared using a lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.5% Triton X-100, supplemented with a cocktail of protease inhibitors (Roche). Protein concentration was determined (Bradford reagent, Bio-Rad) and 30 μg protein was resolved by

SDS–PAGE under reducing conditions using 10% or 4–20% gradient gels. After electrophoresis, proteins were transferred to PVDF membrane (Bio-Rad). The membrane was probed with the appropriate antibody followed by HRP-conjugated secondary antibody and a chemiluminescent substrate (Pierce, Rockford, IL).

Immunoprecipitation. Immunoprecipitation was performed by using the rabbit anti-8 kDa antibody (IgG fraction) that was covalently linked to Sepharose beads. Briefly, 150 μg protein was incubated for 2 h at 4 °C with 10 μl of antibody–Sepharose beads, in 1 ml PBS. The beads were then washed twice with buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) and resuspended in PBS. Sample buffer was then added and after boiling at 100 °C for 5 min, samples were subjected to immunoblot analysis, as described above.

Heparanase activity assay. Preparation of ECM-coated dishes and determination of heparanase activity were performed as described in detail elsewhere [18–20]. Briefly, cell extracts (0.5×10^6 cells/ml) were incubated (24 h, 37 °C, pH 6.2) with ^{35}S-labeled ECM. The incubation medium containing sulfate labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and their radioactivity was counted in a β-scintillation counter. Degradation fragments of HS side chains were eluted at $0.5 < K_{av} < 0.8$ (peak II, fractions 15–30). Nearly intact HSPGs were eluted just after the V_0 ($K_{av} < 0.2$, peak I, fractions 3–15). Each experiment was performed three times and the variation in elution positions (K_{av} values) did not exceed ±15%.

Results

The 8 and 50 kDa heparanase subunits form a heterodimer

A ~50 kDa protein has long been thought to be responsible for heparanase activity detected in various tissues and cells. Indeed, a ~50 kDa band appeared upon processing of the ~65 kDa inactive heparanase precursor into its active form, yet expression of the cloned ~50 kDa protein by itself yielded no enzymatic activity [5–7]. This discrepancy was challenged by Fairbanks et al. [16], who raised the possibility that heparanase exists as a heterodimer composed of the 8 and 50 kDa subunits generated upon heparanase processing. In order to study the existence of heparanase heterodimer and its importance for heparanase enzymatic activity, constructs encoding for each of the heparanase subunits were cloned into the pSecTag2A vector, which directs protein expression in mammalian cells, tagged with the c-Myc epitope at their C-termini.

HEK 293 cells were transfected with the full-length heparanase, the 8, the 50 kDa, or both the 8 and 50 kDa plasmids. Expression levels were examined by immunoblot analysis using anti-c-Myc antibodies. As shown in Fig. 1A, all gene constructs were efficiently expressed and readily detected. In order to evaluate the existence of heterodimer, we employed an immunoprecipitation (IP) analysis, using antibody raised against a peptide located at the C-terminus region of the 8 kDa protein. This antibody (anti-8) specifically recognizes the 8 kDa protein and to a lesser extent the 65 kDa heparanase precursor in immunoblot analysis (Fig. 1B). In addition, we employed antibodies that were raised against a peptide located in the 50 kDa heparanase protein (amino acids 273–291) [16] and recognizing both the 65 and 50 kDa heparanase forms (anti-50, Fig. 1C).

The interaction between the 50 and 8 kDa subunits was first investigated by co-IP experiments. HEK 293 cells expressing the full-length heparanase gene

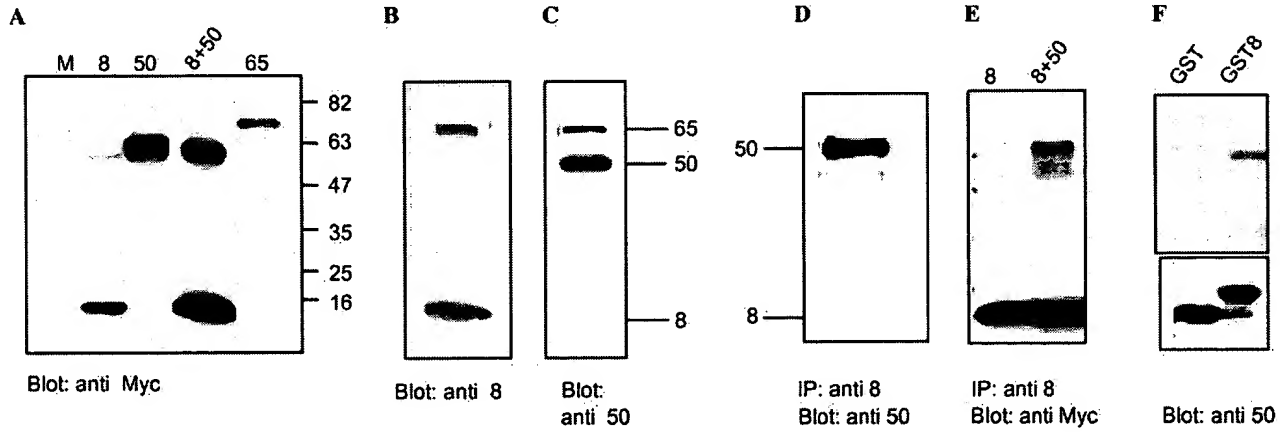

Fig. 1. The 8 and 50 kDa heparanase subunits physically associate. (A) Expression of heparanase subunits in 293 cells. HEK 293 cells were transfected with an empty vector (mock transfected, M), or with plasmids encoding for the 8 kDa (8), 50 kDa (50), both subunits (8 + 50) or the full-length heparanase cDNA (65). Protein expression was evaluated by immunoblot analysis, using anti-c-Myc antibodies. (B–C) Evaluation of anti-heparanase peptide antibodies. 293 cells were transfected with the full-length heparanase cDNA and total cell lysate were subjected to immunoblot analysis with anti-8 kDa (B) or anti-50 kDa (C) antibodies. (D–F) The 8 and the 50 kDa heparanase subunits physically associate. (D) Total cell lysates of 293 cells stably transfected with the full-length heparanase were subjected to IP with anti-8 kDa heparanase antibodies, followed by immunoblotting with anti-50 kDa heparanase antibodies. (E) 293 cells were transfected with the 8 kDa plasmid (8) or co-transfected with the 8 and 50 kDa (50) plasmids. Total cell lysates were subjected to IP with anti-8 kDa antibodies, followed by anti-c-Myc immunoblotting. (F) Pull down experiments. Lysates of 293 cells transfected with the 50 kDa heparanase were incubated (1 h, 4 °C) with bacterially expressed GST alone (GST), or GST-8 kDa fusion protein (GST-8) conjugated to Sepharose beads. After extensive washes, bound proteins were visualized by immunoblotting with anti-50 kDa antibodies (top). Expression of the GST and GST-8 kDa fusion proteins is shown in the lower panel.

construct were extracted and cell lysates were immunoprecipitated (IP) with antibodies raised against the 8 kDa subunit (anti-8), followed by immunoblotting with anti-heparanase antibodies (anti-50). As demonstrated in Fig. 1D, the anti-8 kDa antibodies efficiently precipitated the 50 kDa subunit, confirming the hypothesis that the two subunits generated by heparanase processing form a heterodimer. Similarly, 293 cells expressing the 8 kDa subunit (8) or both the 8 and 50 kDa subunits (8 + 50) were extracted and cell lysates were subjected to IP with an antibody against the 8 kDa subunit, followed by immunoblotting with anti-c-Myc antibodies. As shown in Fig. 1E, antibodies raised against the 8 kDa subunit efficiently precipitated the 50 kDa heparanase subunit in the co-transfected cell extracts, but not in control cells expressing only the 8 kDa protein. This suggests that upon processing of the full-length ~65 kDa heparanase precursor, or by co-expressing subunits, the 8 and 50 kDa proteins heterodimerize and interact with each other.

In addition, heterodimer formation was confirmed by pull down experiments using bacterially expressed GST-8 kDa fusion protein bound to glutathione–agarose beads. HEK 293 cells were transfected with the 50 kDa heparanase subunit and cell extracts were incubated with GST, or with the GST-8 kDa beads. Following extensive washes, bound proteins were analyzed by immunoblotting with anti-heparanase antibody. The 50 kDa heparanase was found to be specifically bound to the GST-8 kDa heparanase fusion protein, but not to the GST control beads (Fig. 1F), further supporting the hypothesis that the 8 and 50 kDa subunits interact with each other and form a heterodimer.

In order to identify protein sequences that mediate the interaction between the two heparanase subunits, we divided the 50 kDa heparanase subunit into three regions of about equal size, representing the N-terminus (Lys^{158}–Gly^{287}), the middle (Glu^{288}–Lys^{417}), and the C-terminus (Val^{418}–Ile^{543}) portions of the 50 kDa protein. All three fragments are abundantly expressed upon transfection into 293 cells (Fig. 2A). Although the three fragments contained a similar number (~130) of amino acids, the N-terminal fragment appears larger on SDS–PAGE due to N-glycosylation sites located in this region. Next, we evaluated the ability of the 8 kDa subunit to interact with each of the 50 kDa fragments. To this end, 293 cells were co-transfected with the 8 kDa plasmid together with each of the 50 kDa fragments and total cell lysates were subjected to IP with the anti-8 kDa antibodies, followed by immunoblotting with anti-C-Myc antibodies. The anti-8 kDa antibodies precipitated the 8 kDa protein in all samples equally well. However, only the middle fragment (Glu^{288}–Lys^{417}) of the 50 kDa protein was co-precipitated with the 8 kDa subunit (Fig. 2B), suggesting that amino acid sequences located in this region are responsible for formation of the heterodimer. We further confirmed this finding by using the GST-8 kDa fusion protein pull down assay. 293 cells were transfected with each of the 50 kDa fragments and total cell lysates were incubated with the GST-8 kDa Sepharose beads. As revealed by the IP experiments (Fig. 2B), only the middle fragment (MD) was pulled down by the GST-8 kDa fusion protein (Fig. 2C).

Heterodimer formation is necessary and sufficient for heparanase enzymatic activity

Heparanase enzymatic activity was evaluated in extracts of 293 cells stably transfected with the different heparanase gene constructs. As demonstrated for transient transfections, the stably transfected 293 cells abundantly express the 8, 50 kDa, or both subunits (Fig. 3A). Cells expressing either the 8 or 50 kDa heparanase subunits did not exhibit any increase in enzymatic

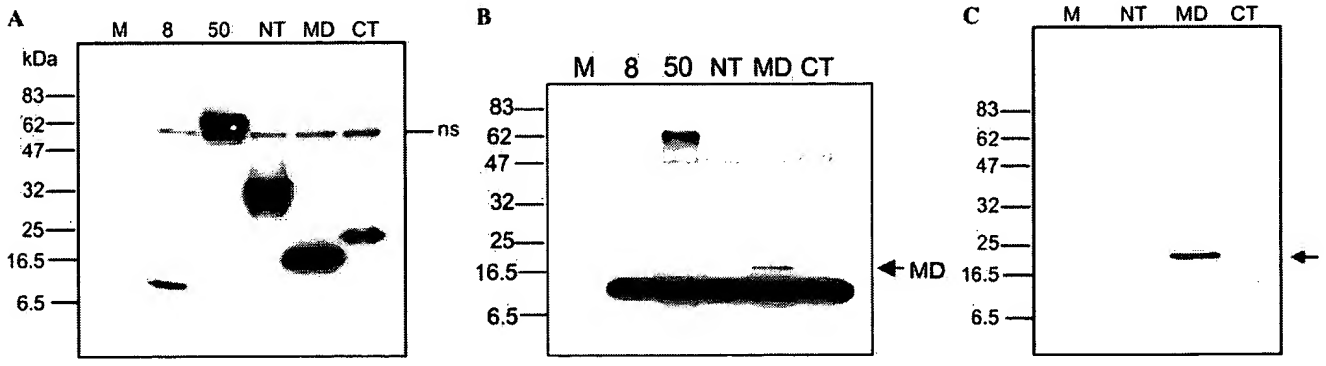

Fig. 2. Identification of a region in the 50 kDa heparanase subunit that interacts with the 8 kDa subunit. 293 cells were transiently transfected with Myc-tagged 8 kDa (8), 50 kDa (50), N-terminal (Lys^{158}–Gly^{287}, NT), middle (Glu^{288}–Lys^{417}, MD), and C-terminal (Val^{418}–Ile^{543}, CT) heparanase gene constructs. Total cell lysates were immunoblotted with anti-c-Myc antibodies (A), or subjected to IP with anti-8 kDa antibodies, followed by anti-c-Myc immunoblotting (B). Lysates of 293 cells transfected with the various gene constructs as in (A) were incubated (1 h, 4 °C) with GST-8 kDa Sepharose beads. Following extensive washing, bound proteins were detected by anti-c-Myc immunoblotting (C). Mock-transfected (M) cells were used as control for antibody specificity. Molecular weight markers are shown on the left. A faint nonspecific (ns) band is seen in the transfected cells.

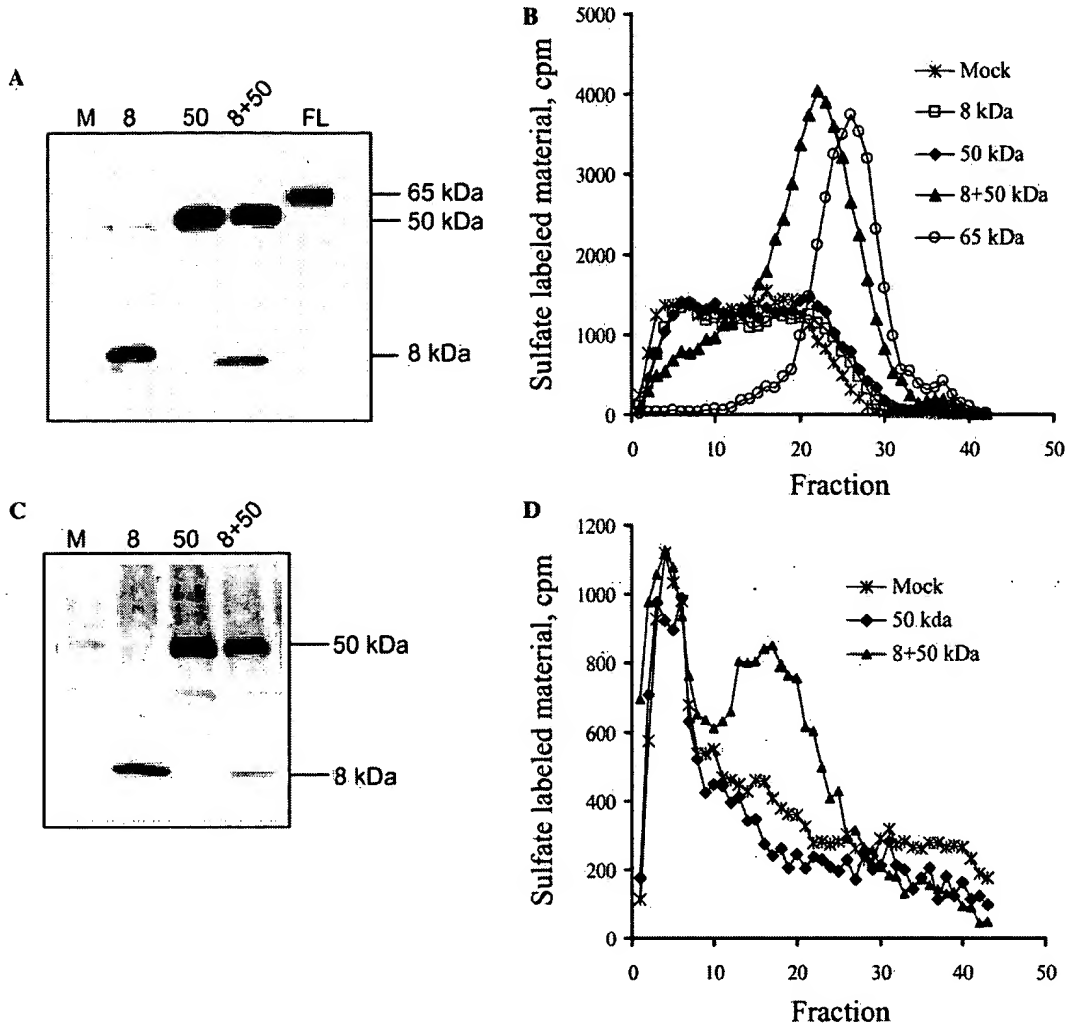

Fig. 3. Heterodimer formation is essential for heparanase enzymatic activity. (A) and (C) Immunoblot analysis of heparanase-transfected cells. 293 (A) and JAR (C) cells were stably transfected with the 8 kDa (8), 50 kDa (50), both the 8 and 50 kDa (8 + 50), or the full-length (FL) heparanase cDNAs. Mock transfected (M) cells were used as control. Cell lysates were subjected to SDS–PAGE and immunoblotting, using anti-c-Myc antibodies. (B) and (D) Heparanase activity. Lysates of 0.5×10^6 293 (B) or JAR (D) cells, mock transfected (✳), or stably transfected with the 8 kDa (□), 50 kDa (◆), both the 8 and 50 kDa (▲), or the full-length heparanase (○) cDNAs were assayed for heparanase activity, as described in Materials and methods. Co-expression of the 8 and 50 kDa heparanase subunits resulted in a gain of heparanase activity, similar to cells transfected with the full-length heparanase cDNA.

activity above the basal level detected in the mock transfected control cells (Fig. 3B). A significant increase in heparanase activity was detected, however, in cell extracts expressing both the 8 and 50 kDa proteins (Fig. 3B). Heparanase activity was similar to that observed in cells transfected with the full-length heparanase cDNA (Fig. 3B). This suggests efficient assembly of the two subunits into an active enzyme. Such acquired heparanase activity upon co-expression of both heparanase subunits was further examined in another tumor-derived cell line. The JAR human choriocarcinoma cells lack heparanase enzymatic activity and exhibit no heparanase mRNA as judged by RT-PCR analysis [21]. JAR cells transfected with the 8, the 50 kDa, or co-transfected with both the 8 and 50 kDa subunits (Fig. 3C) were tested for heparanase enzymatic activity in comparison to mock transfected cells. As was noted with the 293 cells, heparanase activity was detected only in JAR cells expressing both the 50 and 8 kDa heparanase subunits (Fig. 3D). Taken together, our experiments clearly show that the 8 and 50 kDa heparanase subunits heterodimerize and that heterodimer formation is essential for heparanase enzymatic activity.

Discussion

Dynamic interactions between adherent cells and the underlying ECM are crucial for embryonic development, maintenance of cellular functions, and response of

tissues to changes in their immediate extracellular environment, or to insults such as injury and infection. HSPGs play an important structural role in assembly of the ECM. In addition, HSPGs tether growth factors, cytokines, and chemokines to the ECM as high affinity storage depots for bioactive molecules [1–3]. The release of such potent regulators is likely to alter the functional state of cells and tissues and, therefore, must be tightly regulated. HSPG shedding can be catalyzed by proteases, such as thrombin or plasmin, which recognize basic cleavage sites in the HSPG core protein, or by an endoglycosidase, heparanase, which specifically cleaves HS side chains [5,6]. Heparanase is therefore likely to be highly regulated. Induced heparanase expression, documented in a variety of human tumors [9,10,22–25], suggests that regulation at the transcriptional level is likely to occur. Mechanisms that regulate heparanase gene expression are poorly understood, but may involve gene methylation and de-methylation [21,25]. Heparanase processing and localization are two additional major levels of regulation [16,26]. The protease(s) responsible for converting the ~65 kDa inactive heparanase precursor into its ~50 kDa (heterodimer) active form have not been identified. Heparanase activity has been obtained after transfection of mammalian cells with cDNA encoding the full-length heparanase precursor, or after exogenously adding the ~65 kDa heparanase precursor to mammalian cell cultures [27], suggesting that the protease is constitutively active under these experimental conditions. In contrast, transfection of insect cells leads primarily to production of the ~65 kDa heparanase precursor which is not cleaved and lacks enzymatic activity [28]. Heterodimer formation and its necessity for heparanase enzymatic activity provide an additional important level of regulation. Purification of active human [16] and mouse [17] heparanase revealed the presence of a ~50 kDa protein that was accompanied by an 8 kDa protein. This observation led to the hypothesis that active heparanase is a heterodimer in which the 50 kDa protein is non-covalently linked to the 8 kDa protein subunit [16]. We have addressed this hypothesis by determining heparanase activity in mammalian cells transfected with gene constructs encoding the 8, the 50 kDa, or both the 8 and 50 kDa heparanase subunits. Overexpression of each subunit individually had no effect on heparanase enzymatic activity. In contrast, co-transfection with both the 8 and 50 kDa protein subunits resulted in a pronounced increase or gain of heparanase activity in two human cell lines expressing little or no heparanase activity, respectively (Figs. 3B and D). Moreover, by means of immunoprecipitation (Figs. 1D and E) and pull down (Fig. 1F) experiments we were able to clearly demonstrate that the 8 and 50 kDa heparanase subunits physically associate. Such interaction was observed upon processing of the ~65 kDa heparanase precursor (Fig. 1D), as well as in cells co-transfected with the 8 and 50 kDa subunits (Fig. 1E). Moreover, we identified a region in the 50 kDa heparanase subunit (Glu^{288}–Lys^{417}) that mediates the interaction with the 8 kDa subunit (Fig. 2). Additional experiments are underway to identify the minimal sequence required for heterodimer formation. Once identified, such sequences may provide a rational target for the design of heparanase-inhibiting molecules. Importantly, heparanase activity measured after co-transfection of the 8 and 50 kDa subunits was similar in magnitude to that measured in cells transfected with the full-length heparanase (Fig. 1B). This may suggest an efficient association and high affinity interaction between the two subunits. Our results suggest that the linker protein residing between the 8 and 50 kDa heparanase subunits prevents the type of interaction between the two subunits, necessary for formation of an enzymatically active heparanase. McKenzie et al. [28] have very recently reported similar results by co-expressing the 8 and 50 kDa heparanase subunits in insect cells. The results presented in this paper emphasize and further extend the necessity for proper processing and heterodimer formation for heparanase enzymatic activity in mammalian cells.

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